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Suppressor of Cytokine Signaling-1 Is a Critical Regulator of Interleukin-7-Dependent CD8+ T Cell Differentiation

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Summary

To determine the tissue-specific functions of SOCS-1, mice were generated in which the *SOCS-1* gene could be deleted in individual tissues. A reporter gene of SOCS-1 promoter activity was also inserted. Using the reporter, high SOCS-1 expression was found at the $CD4^+CD8^+$ stage in thymocyte development. To investigate the function of this expression, the *SOCS-1* gene was specifically deleted throughout the thymocyte/T/NKT cell compartment. Unlike *SOCS-1*$^{-/-}$ mice, these mice did not develop lethal multiorgan inflammation but developed multiple lymphoid abnormalities, including enhanced differentiation of thymocytes toward $CD8^+$ T cells and very high percentages of peripheral $CD8^+$ T cells with a memory phenotype ($CD44^{hi}CD25^{lo}CD69^{lo}$). These phenotypes were found to correlate with hypersensitivity to the γ-common family of cytokines.

Introduction

Members of the γ-common (γc) family of cytokines, including interleukin (IL)-2, 7, and 15, act on target cells through receptors containing the γc chain. These cytokines signal via the Janus kinase (JAK) signal transducer and activator of transcription (STAT) pathway, activating JAK1, JAK3, STAT5a, and STAT5b (Lin and Leonard, 1997). Studies in humans and mice have demonstrated the critical role γc cytokines play in T cell development. Mutations in any of the genes encoding JAK3, γc, or IL-7 receptor α chain (IL-7Rα) causes severe combined immunodeficiency and a block in early thymocyte development (Leonard, 2001). Targeted gene deletion recapitulates similar phenotypes in mice. IL-7 also appears to have important functions later in thymocyte development and in mature T cells.

Overexpression of suppressor of cytokine signaling-1 (SOCS-1) inhibits JAK-STAT signaling by many cytokines, including IL-2, IL-6, IL-7, and interferons (Alexander, 2002). Because SOCS-1 expression is induced by these cytokines, SOCS-1 is proposed to function in a feedback loop to negatively regulate cytokine signaling. However, it remains uncertain how many of these cytokines that are blocked by SOCS-1 overexpression in vitro are indeed true physiological targets of SOCS-1.

Gene targeting in mice has demonstrated an indispensable role of SOCS-1 for survival. *SOCS-1*$^{-/-}$ mice die by 3 weeks of age with monocytic infiltration and fatty necrosis of the liver, and inflammation of the pancreas and heart (Naka et al., 1998; Starr et al., 1998). These mice also display thymic atrophy and B lymphopenia.

The *SOCS-1*$^{-/-}$ disease is thought to be due to hypersensitivity of tissues to interferon-γ (IFNγ) (Alexander et al., 1999). Neonatal mice injected with IFNγ develop fatty degeneration of the liver and a disease similar to *SOCS-1*$^{-/-}$ mice (Gresser et al., 1981). Furthermore, IFNγ-induced STAT1 activation is prolonged in *SOCS-1*$^{-/-}$ hepatocytes (Brysha et al., 2001), while IFNγ deficiency prevents the neonatal disease in *SOCS-1*$^{-/-}$ mice (Alexander et al., 1999).

SOCS-1 deficiency causes aberrant activation of T cells and NKT cells, which are proposed as the key cellular mediators of the *SOCS-1*$^{-/-}$ disease (Marine et al., 1999; Naka et al., 2001). Removal of lymphocytes by Rag2 deficiency prevents the neonatal lethality in *SOCS-1*$^{-/-}$ mice (Marine et al., 1999). *SOCS-1*$^{-/-}$ mice have increased circulating levels of IFNγ, which also suggests the presence of activated T cells (Marine et al., 1999). In addition, NKT cell depletion reduces the severity of the liver damage in *SOCS-1*$^{-/-}$ mice, whereas NKT cell stimulation accelerates the disease, suggesting that hepatic NKT cells mediate the liver disease (Naka et al., 2001).

The neonatal lethality of the *SOCS-1*$^{-/-}$ disease has complicated the ability to investigate the functions of SOCS-1 in adult tissues. In the present study, the Cre/LoxP system was employed to generated mice in which the *SOCS-1* gene could be conditionally deleted in specific tissues. By deleting *SOCS-1* specifically in the thymocyte/T cell compartment, we show that SOCS-1 has an important function in regulating IL-7-dependent $CD8^+$ T cell differentiation.

Results

The Generation of a Combined SOCS-1 Conditional Deficient/SOCS-1 Reporter Mouse

The endogenous *SOCS-1* (*SOCS-1*$^+$) gene in mice was replaced with a modified *SOCS-1* gene flanked by LoxP sites (*SOCS-1*^{lox}) and a 3′ reporter gene by homologous recombination (Figure 1A). The *SOCS-1*^{lox} allele functionally replaced the endogenous allele because *SOCS-*

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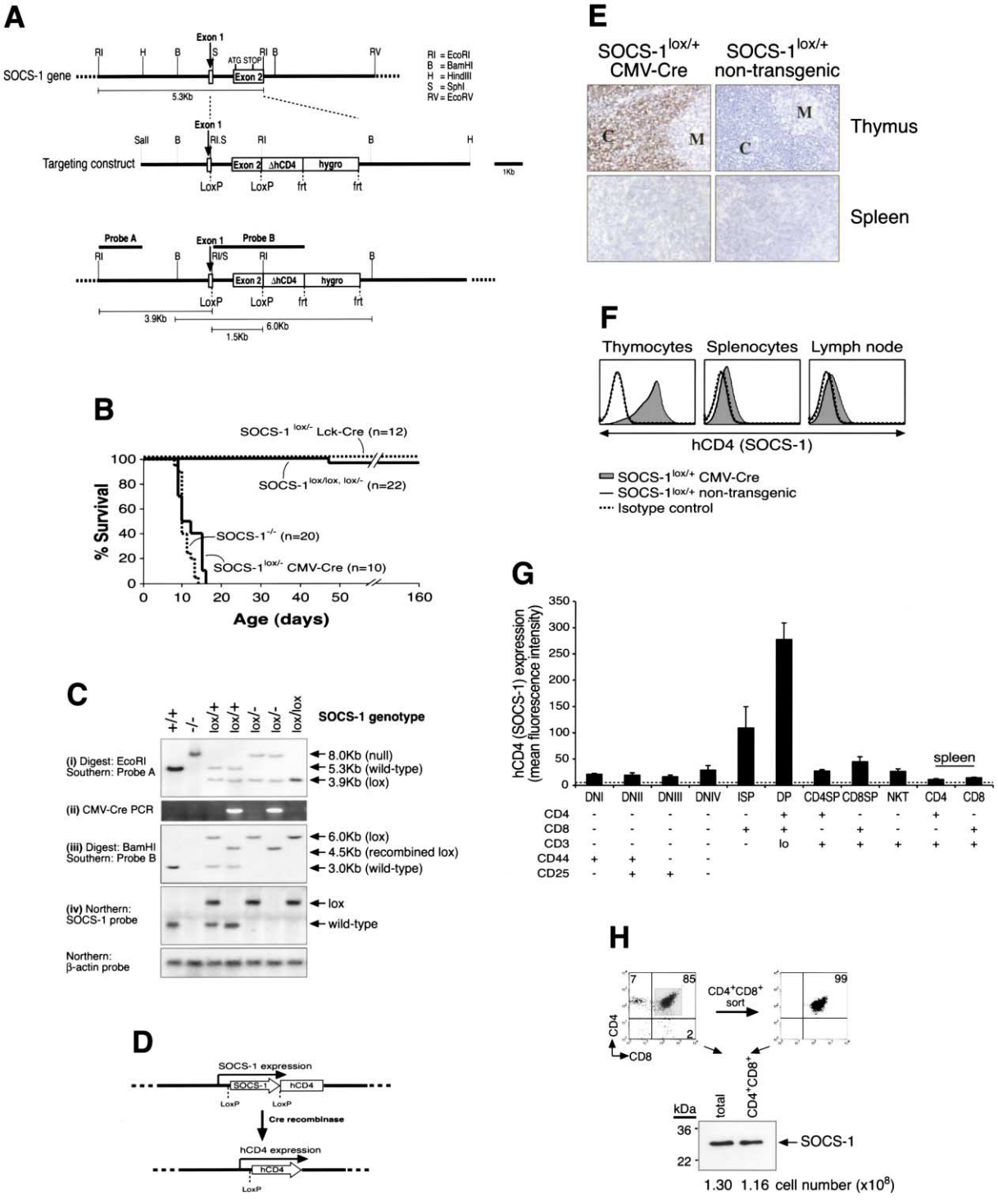

Figure 1. Functional Floxing of the *SOCS-1* Gene in Mice and the Analysis of SOCS-1 Expression during T Cell Development

(A) Shown are the structure of the murine *SOCS-1* locus and the targeting strategy used to flox the SOCS-1 gene.

(B) The *SOCS-1*^{lox} allele functionally replaces the endogenous *SOCS-1* gene in mice. *SOCS-1*$^{lox/lox,lox/-}$ mice are healthy. The *SOCS-1*$^{-/-}$ survival rate is recapitulated in *SOCS-1*$^{lox/-}$ *CMV-Cre* mice but not *SOCS-1*$^{lox/-}$ *Lck-Cre* mice.

(C) CMV-Cre deletes the *SOCS-1*^{lox} allele and mRNA. (i) *SOCS-1* genotyping was performed by Southern analysis of genomic DNA hybridized with probe A (indicated in [A]). (ii) *CMV-Cre* genotyping was performed by PCR. (iii) *SOCS-1*^{lox} deletion confirmed by Southern analysis of genomic DNA hybridized with probe B. Length flanked by the LoxP sites = 1.5 kb. (iv) Loss of SOCS-1 expression from the recombined *SOCS-1*^{lox} allele confirmed by Northern analysis of thymic RNA.

(D) Recombination of the *SOCS-1*^{lox} gene brings the hCD4 reporter under the control of the SOCS-1 promoter.

(E) To examine whether the hCD4 reporter was functional, *SOCS-1*$^{lox/+}$ mice were crossed with *CMV-Cre* mice, which activates the reporter

1$^{lox/lox}$ homozygous or *SOCS-1*$^{lox/-}$ heterozygous mice were healthy and did not develop the *SOCS-1*$^{-/-}$ disease (Figure 1B and data not shown).

To confirm that the LoxP sites were functional, *SOCS-1*$^{lox/+}$ mice were bred with transgenic mice expressing Cre under the CMV promoter (*CMV-Cre*). Because CMV-Cre is expressed early in embryogenesis (Schwenk et al., 1995), any recombination event is transmitted on to all cells in the mouse. The *SOCS-1*^{lox} gene and mRNA were absent from mice that were also *CMV-Cre* transgenic (Figure 1C). Moreover, *SOCS-1*$^{lox/-}$ *CMV-Cre* (*SOCS-1*$^{CMV\text{-}lox}$) mice succumb to the same disease as *SOCS-1*$^{-/-}$ mice (Figure 1B).

SOCS-1 Is Differentially Expressed during T Cell Development

To track the deletion of the *SOCS-1*^{lox} allele in individual cells, a reporter gene was inserted immediately 3′ of the *SOCS-1*^{lox} gene (Figure 1A). Human CD4 (hCD4) containing a F43I mutation and intracellular truncation, which abrogate its function (Sakihama et al., 2000), was employed as the reporter. In the event that the *SOCS-1*^{lox} gene is deleted by Cre, the hCD4 reporter is expressed in place of SOCS-1 (Figure 1D). hCD4 therefore acts both as a marker of *SOCS-1*^{lox} deletion and a reporter of SOCS-1 promoter activity.

SOCS-1$^{lox/+}$ *CMV-Cre* mice, in which all cells carry the recombination event, were analyzed for hCD4 expression. The cortex of the thymus stained strongly for hCD4, while the thymic medulla and the spleen only stained weakly (Figure 1E). The comparatively high level of hCD4 expression in the thymus was also evident by flow cytometry (Figure 1F). The thymus has previously been shown to express high levels of SOCS-1 at the mRNA level (Starr et al., 1997). Nonlymphoid organs did not stain for the reporter (data not shown). hCD4 staining was not detected in the tissues of *SOCS-1*$^{lox/+}$ nontransgenic mice, demonstrating that the reporter is only expressed if the *SOCS-1*^{lox} gene is deleted.

SOCS-1 expression at each stage of thymocyte development was then quantified using the hCD4 reporter (Figure 1G). All CD4$^-$CD8$^-$ double-negative thymocytes (DNI to DNIV) expressed low levels of hCD4. hCD4 expression was upregulated to intermediate levels on CD8$^+$CD3$^-$ immature single-positive (ISP) thymocytes, then to high levels on CD4$^+$CD8$^+$ double-positive (DP) thymocytes. All mature thymocytes, CD4$^+$ single-positive (CD4SP), CD8$^+$ single-positive (CD8SP), and CD3$^+$CD4$^-$CD8$^-$ natural killer T (NKT) cells expressed low levels of hCD4. Importantly, hCD4^{hi} expression was found to correlate with high levels of SOCS-1 protein in DP thymocytes (Figure 1H). This confirms a previous study that reported SOCS-1 protein expression in DP thymocytes (Marine et al., 1999).

Transgenic Expression of Cre under the Lck Promoter Specifically Deletes *SOCS-1*^{lox} in T Cells and NKT Cells

SOCS-1 is expressed at particularly high levels in DP thymocytes. To address the function of this expression, SOCS-1 was deleted from thymocytes and T cells but not other tissues, using Cre expressed under the Lck-promoter (*Lck-Cre*). *SOCS-1*^{lox} recombination in *SOCS-1*$^{lox/+}$ *Lck-Cre* mice was analyzed by tracking hCD4 expression. Individual cell populations from multiple tissues were analyzed. hCD4 was detected on CD4$^+$ and CD8$^+$ T cells, and CD3$^+$NK1.1$^+$ NKT cells but not in NK cells, B cells, monocytes, or granulocytes (Figure 2A and data not shown; only splenocyte data is shown). In addition, IFNγ (which upregulates SOCS-1 promoter activity) only upregulated hCD4 expression on T cells and NKT cells. This contrasts the positive control *SOCS-1*$^{lox/+}$ *CMV-Cre* mice, in which there was hCD4 expression on all populations examined.

SOCS-1^{lox} deletion in both T and NKT cells suggests that Lck-Cre might function at a common developmental stage. Approximately 10% of DNI thymocytes expressed hCD4, which increased to almost 100% in ISP and DP thymocytes (Figure 2B), implying that *SOCS-1*^{lox} deletion by Lck-Cre occurs entirely during thymocyte development. The transmission of *SOCS-1*^{lox} deletion to both T and NKT cells is consistent with previous evidence suggesting that NKT cells are thymocyte derived (Gapin et al., 2001; Pellicci et al., 2002).

Mice with Specific Deletion of SOCS-1 within the Thymocyte/T Cell Compartment Display Abnormal T Cell Population Sizes

Mice with thymocyte/T cell-specific SOCS-1 deficiency (*SOCS-1*$^{Lck\text{-}lox}$) were generated by intercrossing *SOCS-1*$^{+/-}$, *SOCS-1*$^{lox/+}$, and *Lck-Cre* mice together in order to obtain progeny carrying one *SOCS-1* null allele, one *SOCS-1*^{lox} allele, and the *Lck-Cre* transgene (i.e., *SOCS-1*$^{lox/-}$ *Lck-Cre*). With only one *SOCS-1*^{lox} allele requiring recombination, any cell that switched on the hCD4 reporter could be considered deficient in both copies of the *SOCS-1* gene. This breeding strategy also generated littermate *SOCS-1*$^{lox/-}$ nontransgenic genetic controls. Importantly and corresponding to expression of the hCD4 reporter, SOCS-1 protein was absent from the thymocytes of *SOCS-1*$^{Lck\text{-}lox}$ mice but not *SOCS-1*$^{lox/-}$ nontransgenic mice (Figure 2C).

Unlike *SOCS-1*$^{-/-}$ *and SOCS-1*$^{CMV\text{-}lox}$ mice, *SOCS-1*$^{Lck\text{-}lox}$ mice survived past weaning and by 5 months of age still appeared healthy (Figure 1B). However, multiple lymphoid abnormalities became evident. In mice examined at 6 weeks of age, cell counts revealed an increase in the cellularity of some lymphoid organs (Figure 3A). The lymph nodes of *SOCS-1*$^{Lck\text{-}lox}$ mice were enlarged

in all cells. hCD4 expression was examined by immunohistochemistry (brown stain). Shown are the thymus and spleen. C, thymic cortex; M, thymic medulla.

(F) hCD4 expression on thymocytes compared with peripheral lymphocytes measured by flow cytometry.

(G) Thymocyte developmental stages were analyzed for hCD4 staining. The results from four experiments (mean ±SD) are expressed as mean fluorescence intensity. The dashed line indicates the background fluorescence on *SOCS-1*$^{lox/+}$ nontransgenic thymocytes.

(H) The level of SOCS-1 protein expressed in the DP thymocyte population accounts for most of the SOCS-1 protein expressed in total thymocytes.

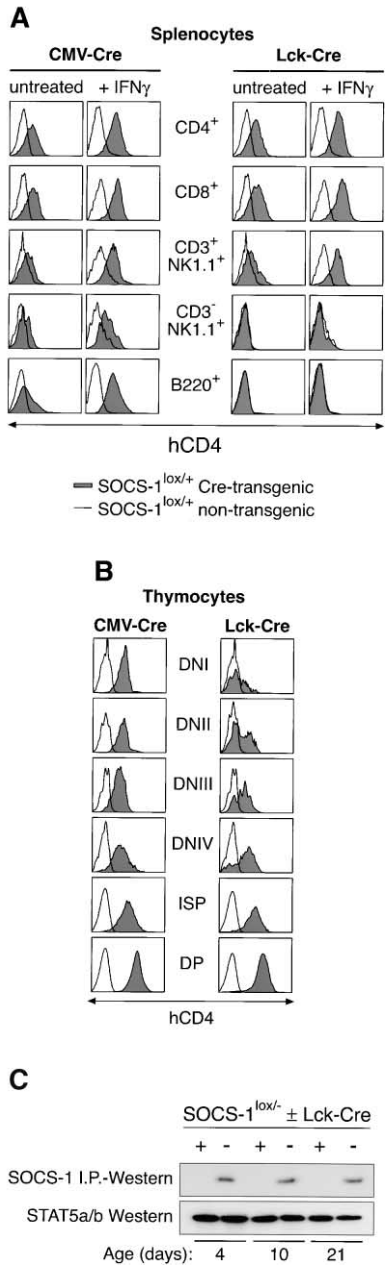

Figure 2. Cre Expressed under the Lck Promoter Deletes *SOCS-1*^{lox} Specifically in T Cells and NKT Cells

Recombination of the *SOCS-1*^{lox} allele in *SOCS-1*$^{lox/+}$ *Lck-Cre* mice was tracked by analyzing hCD4 expression. *SOCS-1*$^{lox/+}$ *CMV-Cre* mice were used as a positive control.

(A) IFNγ-stimulated splenocytes (100 U/ml for 4 hr) were analyzed for hCD4 expression by flow cytometry and costained with the following markers to identify individual populations: CD4, CD4$^+$ T cells; CD8, CD8$^+$ T cells; CD3$^+$NK1.1$^+$, NKT cells; CD$^-$NK1.1$^+$, NK cells; B220, B cells. Monocytes/macrophages and granulocytes from *SOCS-1*$^{lox/+}$ *Lck-Cre* mice were also examined but were found not to express hCD4 (data not shown).

(B) Analysis of Lck-Cre deletion of *SOCS-1*^{lox} during thymocyte development.

(C) *SOCS-1*^{lox} deletion by *Lck-Cre* results in the loss of SOCS-1 protein in thymocytes. Thymocytes from *SOCS-1*$^{lox/-}$ *Lck-Cre* and littermate genetic controls (*SOCS-1*$^{lox/-}$ nontransgenic) were analyzed for SOCS-1 expression. 5 × 10^7 thymocytes were used for each time point. The thymocytes from two mice of the same genotype were pooled for the 4 day time points. As a loading control, 2% of the starting lysate was Western blotted for STAT5a/b.

and contained 2-fold the number of cells compared with littermate controls. There was a marginal but significant increase in the cellularity of the *SOCS-1*$^{Lck\text{-}lox}$ thymus. No difference was found in the spleen. The increased thymic cellularity is in contrast to *SOCS-1*$^{-/-}$ mice, in which there is thymic atrophy due to loss of DP thymocytes (Marine et al., 1999; Starr et al., 1998). Thus, the thymic atrophy in *SOCS-1*$^{-/-}$ mice is likely to be a result of the disease in these animals.

An analysis of thymic populations in *SOCS-1*$^{Lck\text{-}lox}$ mice at 6 weeks of age revealed a large increase in the percentage and number of CD8SP thymocytes (Figure 3B). The preferential increase in CD8SP thymocytes resulted in a marked increase in the ratio of mature CD8SP:CD4SP thymocytes from 1:4 to 1:1. Although the percentage of DP thymocytes was marginally reduced, the absolute of number of DP thymocytes in *SOCS-1*$^{Lck\text{-}lox}$ mice ($1.6 \pm 0.1 \times 10^8$, n = 6) was no different from controls ($1.5 \pm 0.1 \times 10^8$, n = 6). Similarly, there was no change in the size of the DN and ISP populations (data not shown). Mature thymocytes, both CD4SP and CD8SP, in *SOCS-1*$^{Lck\text{-}lox}$ mice were also found to be larger in size (Figure 3C). All *SOCS-1*$^{Lck\text{-}lox}$ mice were found to have enlarged thymic medulla (Figure 3D). This appearance of the medulla may at least be partly a result of the increases in mature thymocyte numbers and cell size. However, these alone do not fully account for the increased medullary region. It is possible that the thymocyte-stroma interaction, which is known to affect stomal development and architecture (Ritter and Boyd, 1993), may also be perturbed in these mice.

The increased cellularity of the lymph nodes in *SOCS-1*$^{Lck\text{-}lox}$ mice was principally due to an increase in the CD8$^+$ T cell population (Figure 3E). Although this was associated with a large reduction in the percentage of non-T cells (mostly of B cells), actual numbers were unchanged. The increased CD8$^+$ T cell numbers in the lymph nodes were also apparent by immunohistochemistry, in which enlargement of T cell areas was evident (Figure 3F).

There were also more CD4$^+$ and CD8$^+$ T cells in the blood (Figure 3E). Although the number of T cells in the spleen was unchanged, there was an increase in the ratio of CD8$^+$:CD4$^+$ in *SOCS-1*$^{Lck\text{-}lox}$ mice (Figure 3E). *SOCS-1*$^{Lck\text{-}lox}$ mice had normal CD3$^+$NK1.1$^+$ NKT cell numbers in the blood and spleen (data not shown). None of these changes in T cell population sizes were due to an immunological response directed against the hCD4 reporter because *SOCS-1*$^{lox/+}$ *LckCre* mice, which also express the hCD4 reporter but have one wild-type allele of *SOCS-1*, were indistinguishable from *SOCS-1*$^{lox/-}$ nontransgenic or *SOCS-1*$^{+/+}$ mice.

T Cell-Specific Deletion of SOCS-1 Results in a Memory Phenotype in CD8$^+$ but Not CD4$^+$ T Cells

Both CD4$^+$ and CD8$^+$ T cells were reported to be aberrantly activated in SOCS-1$^{-/-}$ mice, expressing high levels of the activation markers CD25, CD44, and CD69

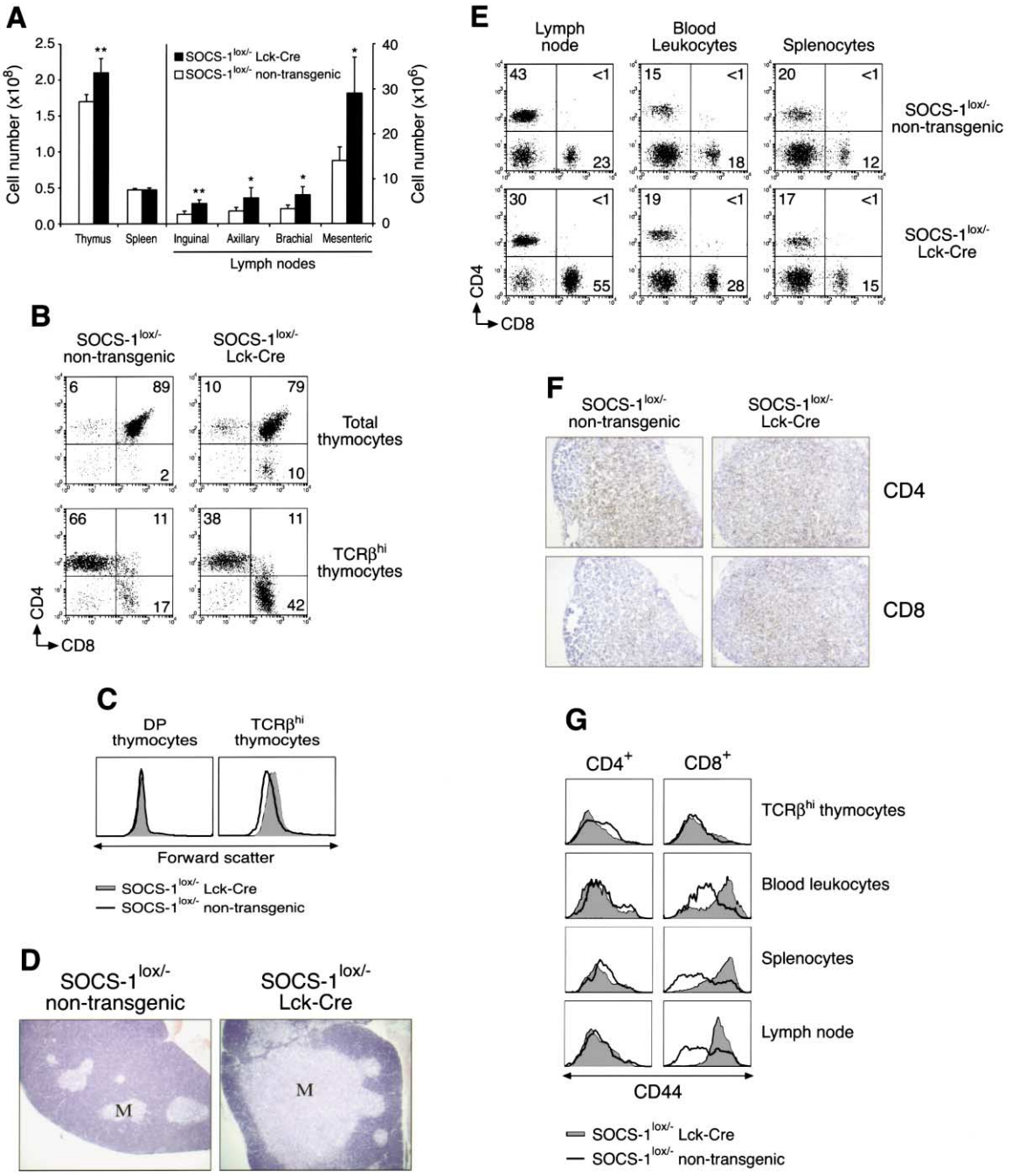

Figure 3. Lymphoid Abnormalities in Mice with Thymocyte/T Cell-Specific SOCS-1 Deficiency

CD8SP thymocyte differentiation is enhanced in *SOCS-1$^{lox/-}$ Lck-Cre* mice compared with littermate controls (*SOCS-1$^{lox/-}$* nontransgenic). For all parameters shown, six pairs of mice at 40 to 45 days of age were analyzed.

(A) The cellularity of the thymus and lymph nodes is increased in *SOCS-1$^{lox/-}$ Lck-Cre* mice. The average cell count of each organ per mouse ±SD is given. Statistical significance: * $p < 0.05$ and ** $p < 0.01$ compared with genetic controls.

(B) Total thymocytes or mature thymocytes (gated on TCRβ^{hi}) were analyzed for CD4 and CD8 expression by flow cytometry.

(C) Mature thymocytes in *SOCS-1$^{lox/-}$ Lck-Cre* mice are larger in size, as indicated by higher forward scatter.

(D) The thymic medulla (M) is enlarged in *SOCS-1$^{lox/-}$ Lck-Cre* mice.

(E) Increased CD8$^+$ T cell populations in *SOCS-1$^{lox/-}$ Lck-Cre* mice are also evident in peripheral lymphoid organs.

(F) Lymph nodes of *SOCS-1$^{lox/-}$ Lck-Cre* mice have enlarged T cell areas. Frozen sections of mesenteric lymph nodes were stained for either CD4 or CD8 (brown stain).

(G) Peripheral CD8$^+$ but not CD4$^+$ T cells in the blood, spleen, and lymph nodes of *SOCS-1$^{lox/-}$ Lck-Cre* mice express high levels of the memory/activation marker CD44.

(Marine et al., 1999). In *SOCS-1$^{Lck\text{-}lox}$* mice, most peripheral CD8$^+$ T cells in the blood, spleen, and lymph nodes expressed high levels of CD44 (Figure 3G) but normal levels of CD25, CD69, and CD62L (data not shown). CD44^{hi}CD25^{lo}CD69^{lo} expression suggests a memory phenotype. There was no abnormal expression of any activation markers on CD4$^+$ T cells in *SOCS-1$^{Lck\text{-}lox}$* mice (Figure 3G, only CD44 data shown). Almost all CD8SP thymocytes in *SOCS-1$^{Lck\text{-}lox}$* mice expressed low levels of CD44, suggesting that the upregulation of CD44 is dependent on a mechanism in the periphery. Therefore, the CD44^{hi} phenotype of CD8$^+$ T cells in *SOCS-1$^{-/-}$* mice is intrinsic to the loss of SOCS-1 within CD8$^+$ T cells, while induction of CD25 and CD69 on CD8$^+$ T cells and the activation of CD4$^+$ T cells are dependent on other factors. This memory phenotype was not due to the hCD4 reporter because it was not evident in *SOCS-1$^{lox/+}$ LckCre* mice, which express hCD4 and have one wild-type allele of the *SOCS-1*. Moreover, *SOCS-1$^{-/-}$IFN$\gamma^{-/-}$* mice, which do not develop the neonatal *SOCS-1$^{-/-}$* disease, also display a similar memory CD8$^+$ T cell phenotype (Cornish et al., 2003).

Although a memory CD8$^+$ T cell phenotype was evident in *SOCS-1$^{Lck\text{-}lox}$* mice, no increase in serum levels of IFNγ was detected. In contrast, *SOCS-1$^{CMV\text{-}lox}$* mice, which develop the typical *SOCS-1$^{-/-}$* disease, had greatly elevated levels of IFNγ in their serum. The serum levels of IFNγ in *SOCS-1$^{CMV\text{-}lox}$* mice were 200 to 800 pg/ml ($n = 5$) compared with $<$50 pg/ml in control mice ($n = 4$) (data not shown). In addition, unlike in *SOCS-1$^{-/-}$* and *SOCS-1$^{CMV\text{-}lox}$* mice, inflammatory lesions were not found in any *SOCS-1$^{Lck\text{-}lox}$* mice at 6 to 8 weeks of age. Therefore, SOCS-1 deficiency in T and NKT cells alone is insufficient to account for the high serum levels of IFNγ and the neonatal disease characteristic of *SOCS-1$^{-/-}$* mice.

SOCS-1-Deficient DP Thymocytes Are Hypersensitive to IL-7-Induced STAT5 Phosphorylation

The positive selection of immature DP thymocytes requires the productive interaction of the TCR with self-MHC to promote survival (Sebzda et al., 1999). The differentiation of DP thymocytes into CD4SP or CD8SP (lineage commitment) requires that the transcription of one coreceptor be terminated (Singer et al., 1999). While this is thought to be dependent on the specificity of the TCR for either class I or class II MHC, cytokines, particularly IL-7, may also be important in driving CD8SP lineage commitment (Brugnera et al., 2000; Varas et al., 1997). Overexpression of SOCS-1 inhibits signaling by cytokines that utilize the JAK-STAT pathway, including IL-7 (Fujimoto et al., 2000). Suppression of IL-7 signaling could be a function of the SOCS-1^{hi} expression in DP thymocytes. SOCS-1 deficiency could cause IL-7 hypersensitivity during DP thymocytes selection and therefore the enhanced CD8SP differentiation seen in *SOCS-1$^{Lck\text{-}lox}$* mice. To examine the effect of SOCS-1 deficiency on IL-7 sensitivity, purified DP thymocytes were stimulated with IL-7, then STAT5 phosphorylation was measured (Figure 4A). IL-7-induced STAT5 phosphorylation in *SOCS-1$^{Lck\text{-}lox}$* DP thymocytes was greatly increased compared to control thymocytes. Little STAT5 phosphorylation was found in SOCS-1-sufficient DP thymocytes. The expression of the IL-7Rα and γc on all thymocyte populations in *SOCS-1$^{Lck\text{-}lox}$* mice was the same as in control mice (Figure 4B and data not shown). Although DP thymocytes expressed low levels of IL-7Rα, as previously described (Brugnera et al., 2000), together with the high levels of γc, this appeared sufficient for IL-7 to signal in the absence of SOCS-1.

SOCS-1-Deficient DP Thymocytes Signaled In Vitro Are Hyperresponsive to IL-7-Induced CD8$^+$ T Cell Differentiation

To investigate whether IL-7 hypersensitivity may be responsible for the enhanced CD8SP differentiation in *SOCS-1$^{Lck\text{-}lox}$* mice, in vitro thymocyte differentiation systems were employed. DP thymocytes "signaled" with PMA and ionomycin to imitate positive selection in vitro can be induced to differentiate into CD8SP cells in the presence of IL-7 (Brugnera et al., 2000). The effect of SOCS-1 deficiency on PMA/ionomycin and IL-7-induced differentiation was examined (Figures 4C–4E). Following overnight culture with PMA/ionomycin, all *SOCS-1$^{Lck\text{-}lox}$* and control DP thymocytes downregulated both CD4 and CD8 to become double-negative. The removal of PMA/ionomycin and the addition of IL-7 resulted in the reexpression of CD4 but not CD8 on half of control thymocytes at day 2. CD8SP cells did not appear in control cultures until day 4. CD8SP cells were generated earlier in *SOCS-1$^{Lck\text{-}lox}$* cultures and in greater percentages at all days compared with control cultures. CD8SP cells were already present in day 2 cultures, with also fewer CD4SP cells. Phenotypically, generated CD8SP cells of both genotypes expressed markers typical of CD8SP thymocytes. CD3 and TCRβ expression were upregulated, and HSA expression was downregulated compared with DP thymocytes (data not shown).

Without the addition of IL-7 after PMA/ionomycin stimulation, CD4SP but not CD8SP cells were generated in both *SOCS-1$^{Lck\text{-}lox}$* and control cultures (data not shown). In addition, treatment with IL-7 alone without PMA/ionomycin did not stimulate the differentiation of DP thymocytes (data not shown).

SOCS-1 Deficiency Increases the Potential of In Vivo Generated CD4$^+$CD8^{lo} (Recently Selected) Thymocytes to Differentiate into CD8SP Cells in Response to IL-7

Because PMA/ionomycin stimulation is an artificial means of positively selecting DP thymocytes, we wanted to analyze DP thymocytes that were recently selected in vivo. Recently selected DP thymocytes have been shown to downregulate CD8 expression regardless of whether they eventually mature into CD4SP or CD8SP (Lundberg et al., 1995; Suzuki et al., 1995). Phenotypically, these thymocytes appear as CD4$^+$CD8^{lo} and have the potential to differentiate into either CD4$^+$ or CD8$^+$ T cells in vivo and in vitro. By measuring hCD4 expression in *SOCS-1$^{lox/+}$ CMV-Cre* mice to quantify SOCS-1 promoter activity, CD4$^+$CD8^{lo} thymocytes were still found to express high levels of SOCS-1 (Figure 5A).

We therefore purified CD4$^+$CD8^{lo} thymocytes and examined the effect of IL-7 on CD4/CD8 expression (Fig-

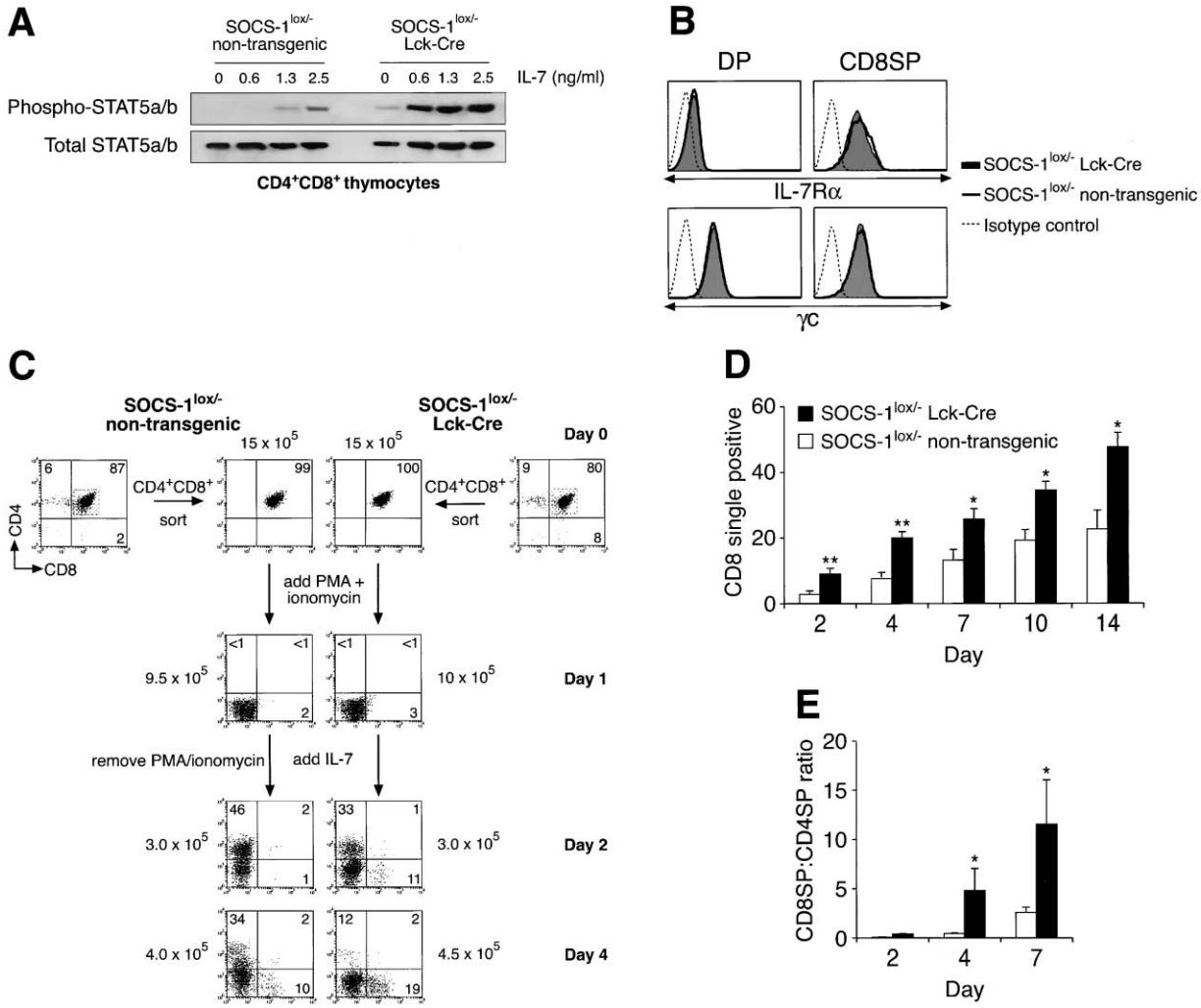

Figure 4. SOCS-1 Deficiency Increases the Potential of PMA/Ionomycin Signaled DP Thymocytes to Differentiate into CD8SP Cells in Response to IL-7

(A) Purified DP thymocytes were treated with 0 to 2.5 ng/ml IL-7 for 30 min; then STAT5 phosphorylation was analyzed by Western blotting. One of three experiments is shown.

(B) IL-7Rα and γc expression levels on DP and CD8SP thymocytes.

(C) Purified DP thymocytes were signaled with 0.5 μM PMA + 0.5 μg/ml ionomycin. After 16 hr, fresh media were replaced with the addition of 2.5 ng/ml IL-7. The cultures were then analyzed for CD4 and CD8 expression over 14 days. Viable cell counts, determined by trypan blue exclusion, are shown adjacent to each dot plot. Shown is the data from days 1 to 4 of one experiment.

(D) The pooled data from four experiments showing the percentage (mean ±SD) of CD8SP cells generated from days 2 to 14.

(E) The pooled data from four experiments showing the ratio (mean ±SD) of CD8SP:CD4SP cells in the days 2 to 14 cultures. Statistical significance: * $p < 0.05$ and ** $p < 0.005$ compared with genetic controls.

ures 5B and 5C). In the absence of IL-7, only CD4SP and DP cells were present in *SOCS-1$^{Lck\text{-}lox}$* and control cultures after 48 hr. In IL-7-treated control cultures, however, one-fifth of cells became CD8SP. Treatment with a combination of IL-7, IL-2, and IL-15 increased the ratio of CD8SP cells to one-third of the culture. One-third of *SOCS-1$^{Lck\text{-}lox}$* cells treated with IL-7 alone appeared as CD8SP, while IL-7/2/15 treatment resulted in two-thirds of cells differentiating into CD8SP. Treatment with either IL-2 or IL-15 alone did not generate CD8SP cells in control cultures but consistently generated a small percentage of CD8SP cells in *SOCS-1$^{Lck\text{-}lox}$* cultures. Phenotypically, both CD4SP and CD8SP cells generated in vitro appeared as CD3^{hi}TCRβ^{hi}HSA^{lo} (data not shown). These results suggest that SOCS-1-deficient DP thymocytes recently selected in vivo are also hyperresponsive to IL-7-induced CD8SP differentiation.

SOCS-1-Deficient Thymocytes Are Normally Deleted in Models of Negative Selection

SOCS-1 could also potentially have a role in regulating negative selection. This was addressed using the Staphylococcal enterotoxin B (SEB) model of negative selection in fetal thymic organ cultures (Aiba et al., 1993). SOCS-1 is expressed in fetal thymic organ cultures (Figure 6A). SEB is a superantigen that engages TCR Vβ8,

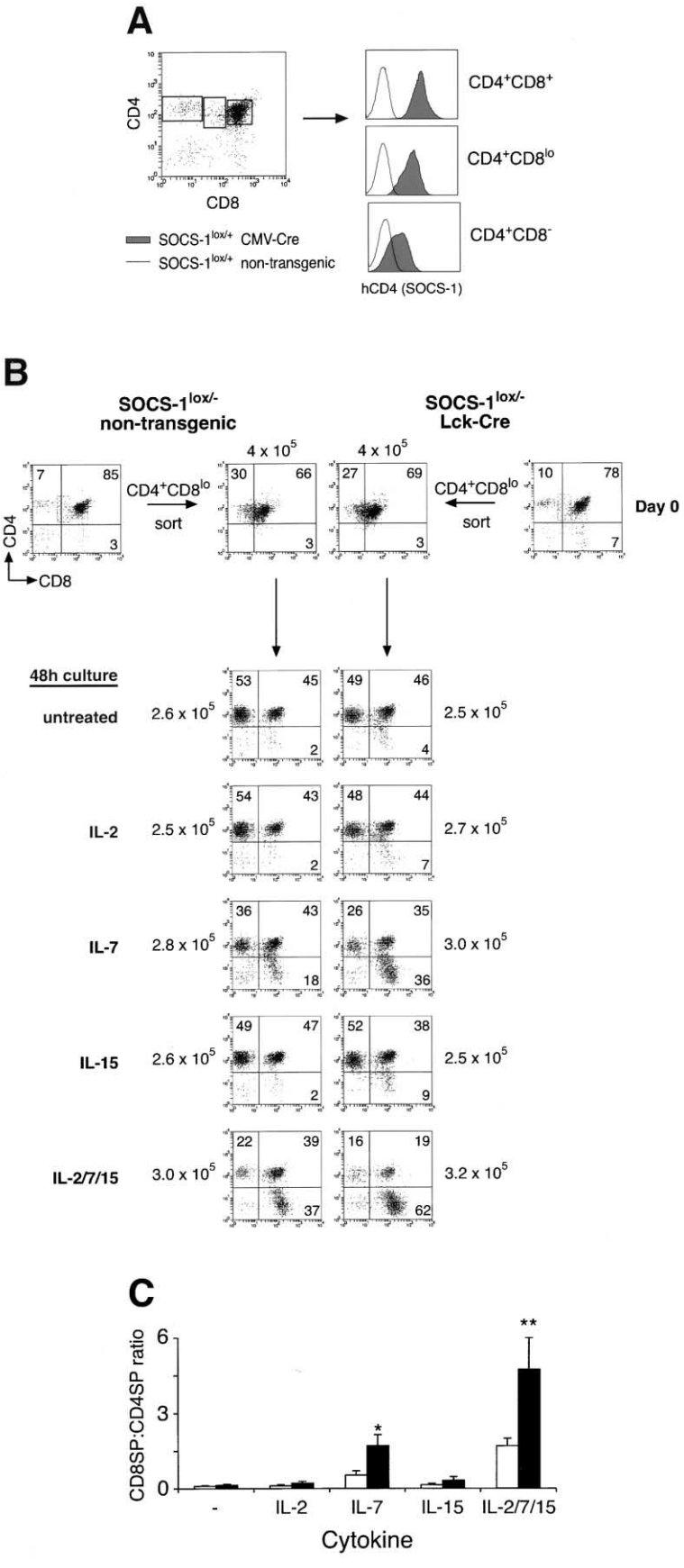

Figure 5. SOCS-1-Deficient DP Thymocytes Positively Selected In Vivo Are Hyperresponsive to IL-7-Induced Differentiation into CD8SP Cells

(A) Recently selected DP thymocytes were identified as $CD4^+CD8^{lo}$ cells. SOCS-1 promoter activity was analyzed by measuring hCD4 expression on *SOCS-1$^{lox/+}$ CMV-Cre* thymocytes.

(B) Purified $CD4^+CD8^{lo}$ thymocytes were cultured with 5 U/ml IL-2, 2.5 ng/ml IL-7, 10 ng/ml IL-15, or all three cytokines for 48 hr. The cells were then analyzed for CD4 and CD8 expression. The viable cell counts, determined by trypan blue exclusion, are adjacent to each dot plot. A representative experiment is shown.

(C) The pooled data from three experiments is shown in (C) as the ratio (mean ±SD) of CD8SP:CD4SP cells generated. Statistical significance: * $p < 0.05$ and ** $p < 0.005$ compared with genetic controls.

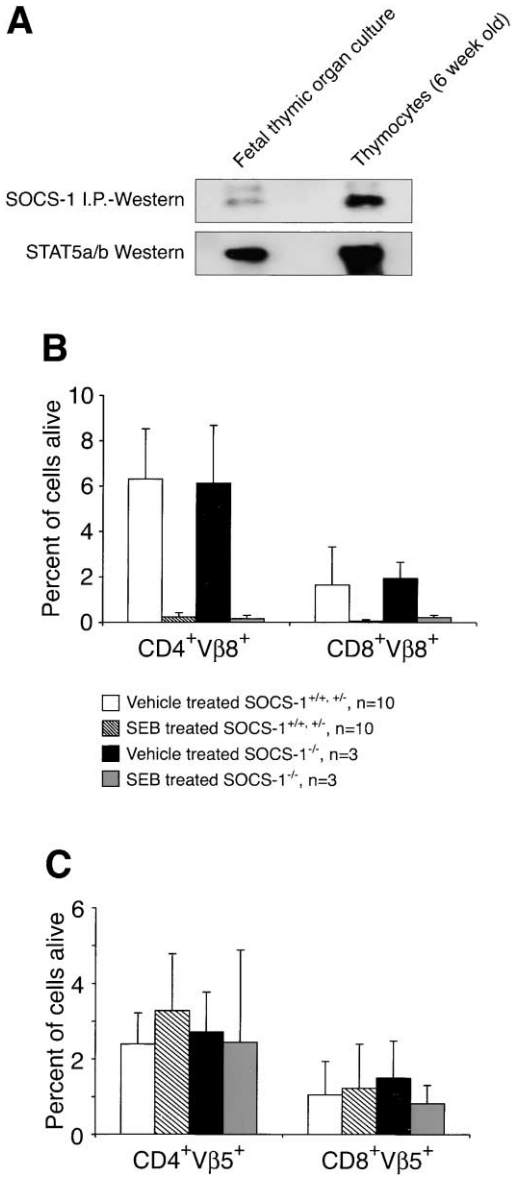

Figure 6. Vβ8+ Thymocyte Deletion in the SEB Model of Negative Selection Is Not Affected by SOCS-1 Deficiency

(A) The thymic lobes from 26 embryos at E15 were cultured for 12 days and then pooled (~3 × 10^6 cells) and analyzed for SOCS-1 protein expression. Thymocytes (2 × 10^7) from a 6-week-old mouse were used a positive control for SOCS-1 expression.

(B) Thymic lobes from E15 *SOCS-1*$^{-/-}$ or *SOCS-1*$^{+/+,+/-}$ littermate embryos were treated with either PBS (vehicle) or SEB. After 12 days, the specific deletion of TCR Vβ8+ thymocytes was determined by flow cytometry. The results are expressed as the percentage (mean ± SD) of live thymocytes that are CD4SP or CD8SP, and Vβ8+.

(C) As a control, the percentage of Vβ5+ thymocytes which should not be deleted was also determined.

resulting in specific deletion of Vβ8+ thymocytes. Equivalent deletion of Vβ8+ thymocytes were found in *SOCS-1*$^{-/-}$ and littermate control (*SOCS-1*$^{+/+,+/-}$) fetal thymic organ cultures (Figure 6A). There was no deletion of Vβ5+ thymocytes in either *SOCS-1*$^{-/-}$ or control cultures (Figure 6B).

The anti-CD3 antibody administration model of negative selection was also examined. *SOCS-1*$^{-/-}$ *IFN*γ$^{-/-}$ mice, which do not develop the neonatal *SOCS-1*$^{-/-}$ disease and thymic atrophy (Alexander et al., 1999), and *IFN*γ$^{-/-}$ mice as controls were injected with anti-CD3 antibody at several doses; then, the deletion of thymocyte populations were analyzed. Similar reductions in thymocytes populations were found in *SOCS-1*$^{-/-}$ *IFN*γ$^{-/-}$ and *IFN*γ$^{-/-}$ mice (data not shown). These results suggest that SOCS-1 is unlikely to have a role in regulating the negative selection of thymocytes.

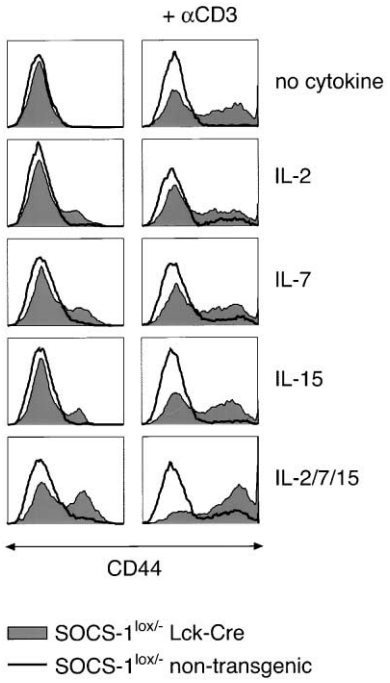

Figure 7. SOCS-1-Deficient Naive CD8+ T Cells Upregulate CD44 Expression in Response to γc Cytokines Alone

Purified mature CD8SP thymocytes were stimulated with 5 U/ml IL-2, 2.5 ng/ml IL-7, or 10 mg/ml IL-15 in the presence or absence of plate-bound anti-CD3 antibody. After 48 hr, the cells were analyzed for CD44 expression. One of four experiments is shown.

SOCS-1-Deficient Naive CD8+ T Cells Are Hyperresponsive to γc Cytokine-Induced CD44 Upregulation

In addition to regulating lineage commitment in the thymus, IL-7 is involved in the generation and maintenance of the memory (CD44^{hi}) CD8+ T cell pool (Schluns et al., 2000). The CD44^{hi} expression on peripheral CD8+ T cells in *SOCS-1*$^{Lck\text{-}lox}$ mice could also be dependent on IL-7 hypersensitivity. To examine this, mature CD8SP thymocytes were purified from *SOCS-1*$^{Lck\text{-}lox}$ mice and stimulated with IL-7 in vitro (Figure 7). The thymus was used as the source of naive CD8+ T cells because very few CD44^{lo} cells were found in the periphery of *SOCS-1*$^{Lck\text{-}lox}$ mice. Treatment with IL-7 alone upregulated CD44 expression on one-fifth of *SOCS-1*$^{Lck\text{-}lox}$ cells, whereas CD44 expression was unchanged in control cells. Other γc cytokines, IL-2 and IL-15, also upregulated CD44 expression on *SOCS-1*$^{Lck\text{-}lox}$ cells. In contrast, upregulation of CD44 on control CD8SP thymocytes required

activation with anti-CD3 in combination with a γc cytokine. Anti-CD3 treatment alone was sufficient to upregulate CD44 expression on *SOCS-1*$^{Lck\text{-}lox}$ cells, but this is probably dependent on the stimulation of endogenous IL-2 production. These results suggest that hypersensitivity to γc cytokines might be responsible for the abnormal memory CD8$^+$ T cell phenotype in *SOCS-1*$^{Lck\text{-}lox}$ mice.

Discussion

SOCS-1 and T Cell Development

In this study, we have generated a combined SOCS-1 conditional deficient/SOCS-1 reporter mouse. The flexibility and sensitivity of the cell surface hCD4 reporter has allowed for more detailed analysis of SOCS-1 promoter activity at the single-cell level than was previously possible. Using this reporter, promoter activity was evident throughout the T cell compartment and at particularly high levels at the DP thymocyte stage, which correlated with SOCS-1 protein expression. This promoter activity in DP thymocytes is unlikely to be dependent on a soluble factor because only low levels were seen in DN thymocytes despite all DN, ISP, and DP thymocytes being present in the thymic cortex. Signaling through TCR is unlikely to cause the high promoter activity either because promoter activity increases from the ISP stage before the upregulation of TCR components. In addition, combinations of cytokines (including IFNγ, IL-2, or IL-7) and TCR activation are unable to achieve the same level of expression in other thymocyte or T cell populations (M.M.W.C. and T.W.H.K., unpublished data). These confirm a previous study which reported that SOCS-1 is still expressed in *Jak3*$^{-/-}$ or *Rag2*$^{-/-}$ thymocytes, which suggests that neither γc cytokine nor TCR signaling is required for this SOCS-1 expression (Marine et al., 1999). Therefore, SOCS-1^{hi} expression at the DP stage is potentially regulated developmentally. Moreover, thymocytes have downregulated expression by the time they are mature TCR^{hi} CD4SP or CD8SP.

SOCS-1^{hi} expression at the DP stage in thymocyte development appears to have a role in regulating IL-7 signaling. *SOCS-1*$^{Lck\text{-}lox}$ mice display a large increase CD8SP thymocyte population and in the ratio of CD8$^+$:CD4$^+$ T cells maturing from the thymus. A preferential increase in the CD8$^+$ T cell population has previously been described in transgenic mice overexpressing IL-7 (Mertsching et al., 1995). Using in vitro systems of thymocyte differentiation, positively selected thymocytes can be preferentially induced to differentiate into CD8SP cells in the presence of IL-7 (Brugnera et al., 2000; Varas et al., 1997). One would expect that the loss of a negative regulator of cytokine signaling, SOCS-1, could result in hyperresponsiveness to IL-7 during thymocyte development. Indeed, we found that loss of SOCS-1 resulted in IL-7 responsiveness (STAT5 phosphorylation) in DP thymocytes, despite the expression of only low levels of IL-7Rα. Furthermore, SOCS-1-deficient DP thymocytes positively selected in vitro or in vivo were hyperresponsive to IL-7-induced differentiation into CD8SP cells.

IL-7 is also important at the earlier stages in thymocyte development because development is blocked at the DN stages in *IL-7*$^{-/-}$ mice (Moore et al., 1996; von Freeden-Jeffry et al., 1995). Therefore, the lower levels of SOCS-1 found in DN thymocytes are unlikely to have significant inhibitory actions on IL-7 signaling. Moreover, there was no evidence of abnormal DN population sizes in *SOCS-1*$^{Lck\text{-}lox}$ mice. In contrast, SOCS-1 overexpression early in thymocyte development inhibits IL-7 signaling and impairs transition from the DNII to DNIII stage (Fujimoto et al., 2000).

Almost all peripheral CD8$^+$ T cells but not CD4$^+$ T cells in *SOCS-1*$^{Lck\text{-}lox}$ mice appeared as CD44^{hi}. This resembles a memory rather than activated phenotype because CD25 or CD69 were not upregulated concurrently. IL-7 and IL-15 are both implicated in the generation and maintenance of the memory CD8$^+$ T cell pool in mice. *IL-15*$^{-/-}$ mice lack memory CD8$^+$ T cells (Kennedy et al., 2000), but this can be overcome by IL-7 overexpression (Kieper et al., 2002). Additionally, memory CD8$^+$ T cells are unable to survive in *IL-7*$^{-/-}$ hosts (Schluns et al., 2000). The high percentage of memory CD8$^+$ but not CD4$^+$ T cells in *SOCS-1*$^{Lck\text{-}lox}$ mice is reminiscent of that seen in mice that transgenically overexpress either IL-7 or IL-15 (Fehniger et al., 2001; Kieper et al., 2002). Therefore, IL-7 and/or IL-15 hypersensitivity could also be the cause of the memory CD8$^+$ T cell phenotype in *SOCS-1*$^{Lck\text{-}lox}$ mice. Indeed, we found that naive CD8$^+$ T cells that lacked SOCS-1 could upregulate CD44 in response IL-7 or IL-15 alone, whereas control cells required the additional signal of anti-CD3 treatment. IL-2 also had the same effect. It has recently been shown that *SOCS-1*$^{-/-}$*Rag1*$^{-/-}$ mice that are transgenic for the OT-1 TCR also display CD44^{hi} expression on peripheral CD8$^+$ T cells (Cornish et al., 2003). Because the CD8$^+$ T cells in these mice only recognize an exogenous antigen, ovalbumin, this suggests that the CD44 upregulation caused by SOCS-1 deficiency is not dependent on TCR signaling. Therefore, SOCS-1 is a critical regulator of signaling by multiple γc cytokines at different stages in CD8$^+$ T cell differentiation.

T Cells and the *SOCS-1*$^{-/-}$ Disease

Disruption of the *SOCS-1* gene in mice has clearly demonstrated its importance for survival. However, the primary cause of the neonatal disease remains unclear. Hypersensitivity of tissues to IFNγ (Alexander et al., 1999) and the abnormal activation of T cells and NKT cells, which in turn mediate the inflammatory disease (Marine et al., 1999; Naka et al., 2001), are two proposed mechanisms of the *SOCS-1*$^{-/-}$ disease. While there is evidence supporting both these hypotheses, the question of which (or both) mechanism has been difficult to address. This is because of the complexity of the *SOCS-1*$^{-/-}$ disease and the likelihood that SOCS-1 deficiency has multiple effects in vivo. By employing the Cre/LoxP system to delete *SOCS-1* in only specific tissues, some of these problems have been circumvented.

SOCS-1 deficiency in the hematopoietic compartment is thought to be sufficient to cause disease because transfer of *SOCS-1*$^{-/-}$ bone marrow into irradiated *Jak3*$^{-/-}$ recipients results in premature lethality (Marine et al., 1999). *SOCS-1*$^{-/-}$ *Rag2*$^{-/-}$ mice do not develop the neonatal *SOCS-1*$^{-/-}$ disease, suggesting that lymphocytes (specifically T cells) are responsible for mediating the *SOCS-1*$^{-/-}$ pathology. In this study, we have

shown that SOCS-1 deficiency in T/NKT cells alone is insufficient to cause the *SOCS-1*$^{-/-}$ disease and neonatal lethality. *SOCS-1*$^{Lck\text{-}lox}$ mice do not develop any of the inflammatory pathologies evident in *SOCS-1*$^{-/-}$ mice. Apart from the CD44^{hi} phenotype of CD8^{+} T cells, little evidence of abnormal T cell activation was found in *SOCS-1*$^{Lck\text{-}lox}$ mice. Therefore, SOCS-1 deficiency in other hematopoietic compartments in addition to T/NKT cells may be required for the lethality observed in *SOCS-1*$^{-/-}$ mice or in mice reconstituted with *SOCS-1*$^{-/-}$ bone marrow. It is clear that SOCS-1 has important functions in other hematopoietic cell types. For example, SOCS-1 regulates the sensitivity of macrophages to lipopolysaccharide (Kinjyo et al., 2002; Nakagawa et al., 2002) and the sensitivity of NK cells to IL-12-induced activation (Eyles et al., 2002). In addition, SOCS-1 deficiency also causes hypersensitivity to other cytokines such as tumor necrosis factor (TNF) (Chong et al., 2002) and IL-4 (Naka et al., 2001).

It was also shown by Marine et al. (1999) that the bone marrow of *SOCS-1*$^{-/-}$ mice contained a large proportion of activated T cells (12% of the total bone marrow). The transfer of activated T cells (as part of the bone marrow transfer) into a lymphopenic environment would result in homeostatic proliferation and activation (Oehen and Brduscha-Riem, 1999; Tanchot et al., 2001). This may have contributed to the disease reported in bone marrow transfers but would not have been a factor in the Cre/LoxP approach used in this study.

Interestingly, in the reported transfer studies, recipient mice did not die until 3 months after receiving *SOCS-1*$^{-/-}$ bone marrow (Marine et al., 1999). This is in contrast to the rapid inflammatory disease that germ-line *SOCS-1*$^{-/-}$ mice develop. This may reflect the time required for bone marrow reconstitution but also suggests that the full-blown *SOCS-1*$^{-/-}$ disease may require deficiency in nonhematopoietic tissues. SOCS-1 deficiency in target tissues alone, such as in hepatocytes, is also insufficient to recapitulate the *SOCS-1*$^{-/-}$ disease (M.M.W.C., unpublished data). The full-blown *SOCS-1*$^{-/-}$ disease could be caused by SOCS-1 deficiency in both T cells and target tissues. The fact that the T cells in *SOCS-1*$^{Lck\text{-}lox}$ mice do not display full activation and do not infiltrate any organs suggests that additional signals are required for T cell activation and infiltration into tissues. Perhaps SOCS-1 deficiency in target tissues causes hypersensitivity to normal circulating levels of cytokines such as IFNγ and TNF, which results in the induction of IFNγ/TNF-regulated chemokines and cytokines, such as IL-7 and IL-15. Many of the tissues that are infiltrated in *SOCS-1*$^{-/-}$ mice, such as the liver, heart, and pancreas, are sites of IL-7 and IL-15 production (Cardozo et al., 2001; Golden-Mason et al., 2001; Grabstein et al., 1994). In addition, IL-7 and IL-15 are stimulators of IFNγ and TNF production by T cells (Borger et al., 1996; McInnes et al., 1997). This positive interaction between IFNγ/TNF and IL-7/15 together with hypersensitivity to all these cytokines could be required for the *SOCS-1*$^{-/-}$ disease.

Experimental Procedures

Generation of *SOCS-1*^{lox} Mice

The genomic murine *SOCS-1* clone 95-11-10 was obtained from Dr. Tracy Willson, The Walter and Eliza Hall Institute. Figure 1A illustrates the structure of the *SOCS-1* gene. The HindIII to EcoRI fragment, containing the *SOCS-1* gene and 1.5 kb of 5′ flanking region, was subcloned into pBluescript. The *SOCS-1* gene was flanked by LoxP sites by inserting the oligonucleotides 5′-CGAATTCCATAACT TCGTATAGCATACATTATACGAAGTTATCGCATG-3′ and 5′-AATT CCATAACTTCGTATAGCATACATTATACGAAGTTATCGGCGCGCC AAGCTTG-3′ into the SphI and EcoRI sites, respectively. An EcoRI site was also inserted with the 5′ LoxP site to allow for genetic screening. A human CD4-SV40 polyA cassette (obtained from Prof. Jerry Adams, The Walter and Eliza Hall Institute) was ligated after the 3′ LoxP site, followed by a PGKhygro cassette (flanked by FRT sites). Finally, the EcoRI to EcoRV fragment (3′ flanking region) from 95-11-10 was ligated after the FRT- PGKhygro-FRT cassette to generate the targeting vector. The vector was targeted into C57BL/6 embryonic stem cells. Two targeted clones were injected into BALB/c blastocysts to generate chimeric mice.

Other Mice and Reagents

SOCS-1$^{-/-}$ mice (Starr et al., 1998), *CMV-Cre* transgenic mice (Schwenk et al., 1995), and *Lck-Cre* transgenic mice (Orban et al., 1992) have been previously described. All mice were backcrossed onto the C57BL/6 background for more than ten generations. The *Lck-Cre* mouse was a kind gift from Dr. Theo Mantamadiotis, The Peter MacCallum Cancer Institute.

The cytokines used were recombinant human IL-2 (McKesson HBOC, Rockville, MD), murine IL-7 (provided by Dr. Stephen Nutt, The Walter and Eliza Hall Institute), recombinant human IL-15 (Peprotech, Rocky Hill, NJ), and recombinant murine IFNγ (Genentech, South San Francisco, CA).

Genotyping of Mice

The *SOCS-1* null, wild-type, and lox alleles were determined by Southern blotting EcoRI-digested genomic DNA with probe A (indicated in Figure 1A). Cre transgenic mice were genotyped by PCR using the primers 5′-AAATTTGCCTGCATTACCGG-3′ and 5′-ACAC CTGCGGTGCTAACCAG-3′.

Recombination of the *SOCS-1*^{lox} allele was examined by Southern blotting BamHI-digested genomic DNA with probe B (indicated in Figure 1A). The 6.0 and 4.5 kb fragments correspond to the *SOCS-1*^{lox} and recombined *SOCS-1*^{lox} alleles, respectively.

Histological Analyses

For histological analyses, tissues were fixed in 10% neutral-buffered formalin and paraffin-embedded, and then sections were stained with hematoxylin and eosin. Tissues for immunohistochemistry were fixed in 4% paraformaldehyde in PBS for 2 hr at 4°C prior to freezing in OCT mounting medium (Sakura Finetechnical, Tokyo, Japan). Sections were stained with monoclonal antibodies (clone name in brackets) recognizing human CD4 (RPA-T4), murine CD4 (H129.19), and murine CD8 (53-6.7) (BD Pharmingen, San Diego, CA).

Flow Cytometry

Monoclonal antibodies recognizing the following antigens (clone name in brackets) were used for flow cytometric analyses: murine CD3 (KT3), CD4 (H129.19), CD8 (53-6.7), CD25 (7D4), CD44 (IM7), CD127/IL-7Rα (B12-1), CD132/γc (4G3), TCRβ (H57-597), TCR Vβ5 (MR9-4), TCR Vβ8 (F23.1), NK1.1 (PK136), and human CD4 (RPA-T4). All antibodies were purchased from BD Pharmingen. Analyses were performed on a LSR or FACStar Plus (BD Immunocytometry). Cell sorting was performed on a MoFlo (Cytomation, Fort Collins, CO).

Immunoprecipitation and Western Blot Analyses

Thymocytes were prepared as single-cell suspensions. SOCS-1 protein expression was measured by immunoprecipiation-Western blotting as previously described (Chong et al., 2001).

STAT5 phosphorylation was analyzed by Western blotting. Protein extracts was obtained by cell lysis as previously described (Chong et al., 2001). The protein extract from 2.5 $\times$ 10^{6} thymocytes was resolved on a 7.5% polyacrylamide gel and transferred to nitrocellulose membrane. The monoclonal antibody ST5P-4A9 was used to detect phosphorylated STAT5a/b. Blots were stripped and reprobed with the monoclonal antibodies ST5a-2H2 and ST5b-10G1 to detect

total STAT5a and b. All anti-STAT5 antibodies were purchased from Zymed Laboratories (South San Francisco, CA).

Thymocyte Suspension and Fetal Thymic Organ Cultures

Thymocyte populations from 6-week-old mice were purified by cell sorting. In vitro differentiation of $CD4^+CD8^+$ thymocytes was performed according to the method of Brugnera et al. (2000). Over a 2 week period, the cells were harvested, and cell viability was determined by trypan blue exclusion and counting on a hemocytometer; then analyzed by flow cytometry. Other thymocyte populations were also cultured with various cytokines prior to analysis by flow cytometry. In some experiments, cells were also stimulated with plate-bound anti-CD3 (145-2C11, produced in-house).

Fetal thymic organ cultures were performed on embryonic day 15 thymic lobes as previously described (Godfrey et al., 1994). They were cultured for 12 days with or without 10 μg/ml SEB (Toxin Technology, Sarasota, FL); then the thymocytes were analyzed by flow cytometry.

Statistical Analyses

Statistical comparisons made using the Mann-Whitney test calculated with Prism version 2.0a (GraphPad Software Inc., San Diego, CA).

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References

Aiba, Y., Mazda, O., Matsuzaki, Y., Nakauchi, H., Muramatsu, S., and Katsura, Y. (1993). Clonal deletion of thymic mature T cells induced by staphylococcal enterotoxin B in murine fetal thymus organ culture. Eur. J. Immunol. *23*, 815–819.

Alexander, W.S. (2002). Suppressors of cytokine signalling (SOCS) in the immune system. Nat. Rev. Immunol. *2*, 410–416.

Alexander, W.S., Starr, R., Fenner, J.E., Scott, C.L., Handman, E., Sprigg, N.S., Corbin, J.E., Cornish, A.L., Darwiche, R., Owczarek, C.M., et al. (1999). SOCS1 is a critical inhibitor of interferon γ signaling and prevents the potentially fatal neonatal actions of this cytokine. Cell *96*, 597–608.

Borger, P., Kauffman, H.F., Postma, D.S., and Vellenga, E. (1996). IL-7 differentially modulates the expression of IFN-γ and IL-4 in activated human T lymphocytes by transcriptional and post-transcriptional mechanisms. J. Immunol. *156*, 1333–1338.

Brugnera, E., Bhandoola, A., Cibotti, R., Yu, Q., Guinter, T.I., Yamashita, Y., Sharrow, S.O., and Singer, A. (2000). Coreceptor reversal in the thymus: signaled $CD4^+8^+$ thymocytes initially terminate CD8 transcription even when differentiating into $CD8^+$ T cells. Immunity *13*, 59–71.

Brysha, M., Zhang, J.G., Bertolino, P., Corbin, J.E., Alexander, W.S., Nicola, N.A., Hilton, D.J., and Starr, R. (2001). Suppressor of cytokine signalling-1 attenuates the duration of interferon γ signal transduction in vitro and in vivo. J. Biol. Chem. *276*, 22086–22089.

Cardozo, A.K., Heimberg, H., Heremans, Y., Leeman, R., Kutlu, B., Kruhoffer, M., Orntoft, T., and Eizirik, D.L. (2001). A comprehensive analysis of cytokine-induced and nuclear factor-κB-dependent genes in primary rat pancreatic β-cells. J. Biol. Chem. *276*, 48879–48886.

Chong, M.M.W., Thomas, H.E., and Kay, T.W.H. (2001). Interferon-γ signaling in pancreatic β cells is persistent but can be terminated by overexpression of suppressor of cytokine signaling-1. Diabetes *50*, 2744–2751.

Chong, M.M.W., Thomas, H.E., and Kay, T.W.H. (2002). Suppressor of cytokine signaling-1 regulates the sensitivity of pancreatic β cells to tumor necrosis factor. J. Biol. Chem. *277*, 27945–27952.

Cornish, A.L., Davey, G.M., Metcalf, D., Purton, J.F., Corbin, J.E., Greenhalgh, C.J., Darwiche, R., Wu, L., Nicola, N.A., Godfrey, D.I., et al. (2003). Suppressor of cytokine signaling-1 has IFN-γ-independent actions in T cell homeostasis. J. Immunol. *170*, 878–886.

Eyles, J.L., Metcalf, D., Grusby, M.J., Hilton, D.J., and Starr, R. (2002). Negative regulation of interleukin-12 signaling by suppressor of cytokine signaling-1. J. Biol. Chem. *277*, 43735–43740.

Fehniger, T.A., Suzuki, K., Ponnappan, A., VanDeusen, J.B., Cooper, M.A., Florea, S.M., Freud, A.G., Robinson, M.L., Durbin, J., and Caligiuri, M.A. (2001). Fatal leukemia in interleukin 15 transgenic mice follows early expansions in natural killer and memory phenotype CD8+ T cells. J. Exp. Med. *193*, 219–231.

Fujimoto, M., Naka, T., Nakagawa, R., Kawazoe, Y., Morita, Y., Tateishi, A., Okumura, K., Narazaki, M., and Kishimoto, T. (2000). Defective thymocyte development and perturbed homeostasis of T cells in STAT-induced STAT inhibitor-1/suppressors of cytokine signaling-1 transgenic mice. J. Immunol. *165*, 1799–1806.

Gapin, L., Matsuda, J.L., Surh, C.D., and Kronenberg, M. (2001). NKT cells derive from double-positive thymocytes that are positively selected by CD1d. Nat. Immunol. *2*, 971–978.

Godfrey, D.I., Kennedy, J., Gately, M.K., Hakimi, J., Hubbard, B.R., and Zlotnik, A. (1994). IL-12 influences intrathymic T cell development. J. Immunol. *152*, 2729–2735.

Golden-Mason, L., Kelly, A.M., Traynor, O., McEntee, G., Kelly, J., Hegarty, J.E., and O'Farrelly, C. (2001). Expression of interleukin 7 (IL-7) mRNA and protein in the normal adult human liver: implications for extrathymic T cell development. Cytokine *14*, 143–151.

Grabstein, K.H., Eisenman, J., Shanebeck, K., Rauch, C., Srinivasan, S., Fung, V., Beers, C., Richardson, J., Schoenborn, M.A., and Ahdieh, M. (1994). Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. Science *264*, 965–968.

Gresser, I., Aguet, M., Morel-Maroger, L., Woodrow, D., Puvion-Dutilleul, F., Guillon, J.C., and Maury, C. (1981). Electrophoretically pure mouse interferon inhibits growth, induces liver and kidney lesions, and kills suckling mice. Am. J. Pathol. *102*, 396–402.

Kennedy, M.K., Glaccum, M., Brown, S.N., Butz, E.A., Viney, J.L., Embers, M., Matsuki, N., Charrier, K., Sedger, L., Willis, C.R., et al. (2000). Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J. Exp. Med. *191*, 771–780.

Kieper, W.C., Tan, J.T., Bondi-Boyd, B., Gapin, L., Sprent, J., Ceredig, R., and Surh, C.D. (2002). Overexpression of interleukin (IL)-7 leads to IL-15-independent generation of memory phenotype CD8+ T cells. J. Exp. Med. *195*, 1533–1539.

Kinjyo, I., Hanada, T., Inagaki-Ohara, K., Mori, H., Aki, D., Ohishi, M., Yoshida, H., Kubo, M., and Yoshimura, A. (2002). SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. Immunity *17*, 583–591.

Leonard, W.J. (2001). Cytokines and immunodeficiency diseases. Nat. Rev. Immunol. *1*, 200–208.

Lin, J.X., and Leonard, W.J. (1997). Signaling from the IL-2 receptor to the nucleus. Cytokine Growth Factor Rev. *8*, 313–332.

Lundberg, K., Heath, W., Kontgen, F., Carbone, F.R., and Shortman, K. (1995). Intermediate steps in positive selection: differentiation of CD4+8int TCRint thymocytes into CD4–8+TCRhi thymocytes. J. Exp. Med. *181*, 1643–1651.

Marine, J.-C., Topham, D.J., McKay, C., Wang, D., Parganas, E., Stravopodis, D., Yoshimura, A., and Ihle, J.N. (1999). SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality. Cell *98*, 609–616.

McInnes, I.B., Leung, B.P., Sturrock, R.D., Field, M., and Liew, F.Y. (1997). Interleukin-15 mediates T cell-dependent regulation of tumor necrosis factor-α production in rheumatoid arthritis. Nat. Med. *3*, 189–195.

Mertsching, E., Burdet, C., and Ceredig, R. (1995). IL-7 transgenic

mice: analysis of the role of IL-7 in the differentiation of thymocytes in vivo and in vitro. Int. Immunol. *7*, 401–414.

Moore, T.A., von Freeden-Jeffry, U., Murray, R., and Zlotnik, A. (1996). Inhibition of γδ T cell development and early thymocyte maturation in IL-7 −/− mice. J. Immunol. *157*, 2366–2373.

Naka, T., Matsumoto, T., Narazaki, M., Fujimoto, M., Morita, Y., Ohsawa, Y., Saito, H., Nagasawa, T., Uchiyama, Y., and Kishimoto, T. (1998). Accelerated apoptosis of lymphocytes by augmented induction of Bax in SSI-1 (STAT-induced STAT inhibitor-1) deficient mice. Proc. Natl. Acad. Sci. USA *95*, 15577–15582.

Naka, T., Tsutsui, H., Fujimoto, M., Kawazoe, Y., Kohzaki, H., Morita, Y., Nakagawa, R., Narazaki, M., Adachi, K., Yoshimoto, T., et al. (2001). SOCS-1/SSI-1-deficient NKT cells participate in severe hepatitis through dysregulated cross-talk inhibition of IFN-γ and IL-4 signaling in vivo. Immunity *14*, 535–545.

Nakagawa, R., Naka, T., Tsutsui, H., Fujimoto, M., Kimura, A., Abe, T., Seki, E., Sato, S., Takeuchi, O., Takeda, K., et al. (2002). SOCS-1 participates in negative regulation of LPS responses. Immunity *17*, 677–687.

Oehen, S., and Brduscha-Riem, K. (1999). Naive cytotoxic T lymphocytes spontaneously acquire effector function in lymphocytopenic recipients: a pitfall for T cell memory studies? Eur. J. Immunol. *29*, 608–614.

Orban, P.C., Chui, D., and Marth, J.D. (1992). Tissue- and site-specific DNA recombination in transgenic mice. Proc. Natl. Acad. Sci. USA *89*, 6861–6865.

Pellicci, D.G., Hammond, K.J., Uldrich, A.P., Baxter, A.G., Smyth, M.J., and Godfrey, D.I. (2002). A natural killer T (NKT) cell developmental pathway involving a thymus-dependent NK1.1(−)CD4(+) CD1d-dependent precursor stage. J. Exp. Med. *195*, 835–844.

Ritter, M.A., and Boyd, R.L. (1993). Development in the thymus: it takes two to tango. Immunol. Today *14*, 462–469.

Sakihama, T., Hunsicker, M.E., Hussey, R.E., and Reinherz, E.L. (2000). Human CD4 residue Phe 43 is critical for repertoire development and maturation of MHC class II restricted CD4 single-positive T lineage cells in vivo. Eur. J. Immunol. *30*, 279–290.

Schluns, K.S., Kieper, W.C., Jameson, S.C., and Lefrancois, L. (2000). Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. Nat. Immunol. *1*, 426–432.

Schwenk, F., Baron, U., and Rajewsky, K. (1995). A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. Nucleic Acids Res. *23*, 5080–5081.

Sebzda, E., Mariathasan, S., Ohteki, T., Jones, R., Bachmann, M.F., and Ohashi, P.S. (1999). Selection of the T cell repertoire. Annu. Rev. Immunol. *17*, 829–874.

Singer, A., Bosselut, R., and Bhandoola, A. (1999). Signals involved in CD4/CD8 lineage commitment: current concepts and potential mechanisms. Semin. Immunol. *11*, 273–281.

Starr, R., Willson, T.A., Viney, E.M., Murray, L.J., Rayner, J.R., Jenkins, B.J., Gonda, T.J., Alexander, W.S., Metcalf, D., Nicola, N.A., and Hilton, D.J. (1997). A family of cytokine-inducible inhibitors of signalling. Nature *387*, 917–921.

Starr, R., Metcalf, D., Elefanty, A.G., Brysha, M., Willson, T.A., Nicola, N.A., Hilton, D.J., and Alexander, W.S. (1998). Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine signaling-1. Proc. Natl. Acad. Sci. USA *95*, 14395–14399.

Suzuki, H., Punt, J.A., Granger, L.G., and Singer, A. (1995). Asymmetric signaling requirements for thymocyte commitment to the CD4+ versus CD8+ T cell lineages: a new perspective on thymic commitment and selection. Immunity *2*, 413–425.

Tanchot, C., Le Campion, A., Leaument, S., Dautigny, N., and Lucas, B. (2001). Naive CD4(+) lymphocytes convert to anergic or memory-like cells in T cell-deprived recipients. Eur. J. Immunol. *31*, 2256–2265.

Varas, A., Vicente, A., Jimenez, E., Alonso, L., Moreno, J., Munoz, J.J., and Zapata, A.G. (1997). Interleukin-7 treatment promotes the differentiation pathway of T-cell-receptor-αβ cells selectively to the CD8+ cell lineage. Immunology *92*, 457–464.

von Freeden-Jeffry, U., Vieira, P., Lucian, L.A., McNeil, T., Burdach, S.E., and Murray, R. (1995). Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J. Exp. Med. *181*, 1519–1526.